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(54) Title: HIGH EFFICIENCY PACKAGING OF MUTANT ADENO-ASSOCIATED VIRUS USING AMBER SUPPRESSIONS

(57) Abstract

Described is the construction of an adeno-associated virus (AAV) plasmid containing an amber mutation within the AAV *rep* gene and the propagation of this mutant AAV on monkey cell lines containing an amber suppressor. It is shown that pure populations of AAV particles containing the mutant genome could be generated. The spontaneous reversion frequency of the amber mutation to wild-type was less than 10^{-5}. This reversion frequency was significantly elevated by growth of the mutant virus in cultured human cells treated with mutagens. The amber mutant AAV particles provide a novel and sensitive assay for mutagenic agents in cultured human cells.

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HIGH EFFICIENCY PACKAGING OF MUTANT ADENO-ASSOCIATED VIRUS USING AMBER SUPPRESSION

Described herein is the construction of an adeno-associated virus (AAV) plasmid containing an amber mutation within the AAV rep gene and the propagation of this mutant AAV on monkey cell lines containing an amber suppressor. It is shown that pure populations of AAV particles containing the mutant genome could be generated. The spontaneous reversion frequency of the amber mutation to wild-type was less tan 10^{-5}. This reversion frequency was significantly elevated by growth of the mutant virus in cultured human cells treated with mutagens. The amber mutant AAV particles provide a novel and sensitive assay for mutagenic agents in cultured human cells.

SUMMARY OF THE INVENTION

When recombinant plasmids containing the entire adeno-associated virus genome are transfected into permissive cells infected with a helper adenovirus, infectious AAV particles are efficiently generated. These plasmids can be used to generate mutant AAV genomes or recombinant AAV vectors. Packaging of mutant AAV genomes has required complementation with a second AAV plasmid in the transfection assay which may lead to generation of significant amounts of wild-type AAV recombinants. One approach to alliviate production of wild-type recombinants was to generate conditional lethal mutants. We constructed an AAV-plasmid recombinant having a nonsense mutation in the AAV rep gene by using oligonucleotide-directed mutagenesis to convert a serine codon to an amber codon. We show that this mutant AAV can be grown on monkey cell lines containing an inducible human serine tRNA amber

suppressor. The amber suppression is efficient and yields a burst of mutant AAV particles at about 10% of the titer of wild type AAV. Subsequent infection of non-suppressor cells with the mutant AAV showed that the spontaneous reversion frequency of the amber mutation was less than 10^{-5}. This reversion frequency was significantly enhanced by growth of the mutant virus in cultured human cells treated with mutagens. This provides a novel and sensitive assay to analyse mutagenic agents in human cells.

BACKGROUND OF THE INVENTION

Genetic analysis in many systems has been greatly advanced by the exploitation of conditional-lethal mutations such as temperature-sensitivity or host-range dependence. Also suppression of nonsense mutations by suppressor tRNAs has been very important in prokaryotes and lower eukaryotes.

Recently, Sedivy et al., (Cell, 1987, 50:379) constructed monkey cell lines having an inducible human amber suppressor $tRNA^{ser}$ gene contained in an SV40 replicon. These cells also contain a temperature-sensitive SV40 T-antigen gene. Upon shift-down from non-permissive to permissive temperature, the suppressor tRNA gene is amplified allowing suppression of amber condons. These cells were used to propagate a poliovirus containing an amber mutation in its replicase gene. We have examined the use of these cell lines for propagation of amber mutation of adeno-associated virus type 2 (AAV2).

The AAV2 genome cloned into recombinant bacterial plasmids is readily infectious. Transfection of recombinant AAV-plasmid DNA into adenovirus-infected

cells results in efficient rescue of the AAV genome and replication free of plasmid to yield infectious AAV particles (Samulski et al., Proc. Natl. Acad. Sci. USA, 1982 79:2077; Laughlin et al., Gene, 1983, 23:65). In vitro mutagenesis of recombinant AAV-plasmids has provided an approach to a genetic analysis of AAV (Tratschin et al., J. Virol., 1984, 51:611; Hermonat et al., J. Virol., 1984, 51:329 and to generation of AAV vectors for introduction and expression of foreign genes in mammalian cells (Hermonat and Muzyczka, Proc. Natl. Acad. Sci. USA, 1984, 81:6466; Tratschin et al., Mol. cell. Biol., 1984, 4:2072 and 1985, 5:3251; McLaughlin et al., J. Virol., 1988, 62:1963; Lebkowski et al., Mol. Cell. Biol., 1988 8:3998). This genetic analysis requires introduction of mutant genomes via DNA transfection which may have physiological differences to normal viral growth following infection with AAV particles. Mutant AAV genomes or AAV vectors can be packaged into virus particles by complementation in the transfection assay but this often leads to generation of significant amounts of wild type genomes or low titers of the packaged mutant which may complicate both genetic analysis and certain other uses of AAV vectors.

A significant obstacle in studies on AAV and the development of AAV vectors has been the difficulty in generating high titer stocks of particles containing defined mutant genomes or AAV vectors free of wild type contamination. The generation of recombinant plasmids containing the entire AAV genome (Samulski et al., Proc. Natl. Acad. Sci. USA., 1982, 79:2077; Laughlin et al., Gene, 1983 23:65) facilitated construction of

defined mutants or vectors which could be analyzed by DNA transfection of permissive cells (Hermonat et al., J. Virol., 1984, 57:329; Tratschin et al., J. Virol., 1984, 51:611). However, this leads to uncertainties concerning the accuracy with which events after DNA transfection reflect those events which occur in a normal infection with AAV particles. The mutant AAV genomes from plasmids can be packaged into AAV particles by cotransfection with appropriate complementing plasmids expressing the AAV rep or cap genes (Hermonat and Muzyczka, Proc. Natl. Acad. Sci. USA, 1984, 81:6466; Tratschin et al., Mol. Cell. Biol., 1985, 5:3251; Lebkowski et al., Mol. Cell. Biol., 1988, 8:3988; McLaughlin et al., J. Virol., 1988 62:1963). However, the titer of such mutant particle preparations was generally low. In addition significant levels of wild type recombinants were often generated even when there was no direct AAV DNA sequence homology between the complementing AAV plasmids (Senapathy and Carter, J. Biol. Chem., 1984, 259:4661). The presence of the wild type recombinants has prevented efficient genetic analysis of AAV via particle-mediated transfections and has compromised the efficient use of AAV vectors. For AAV vectors packaged into transdusing particles the presence of wild type genomes is an additional problem because of the strong negative regulatory effect of rep on expression in many cell types (Tratschin et al., Mol. Cell. Biol., 1986, 6:2884; Lebow et al., Mol. Cell. Biol., 1987, 7:1320; McLaughlin et al., J. Virol., 1988, 62:1963; Mendelson et al., Virology, 1988, 166:612).

One approach to these problems is to generate conditional lethal mutations in an essential AAV gene. It was an object of the present invention to construct an amber mutation in the AAV rep gene to generate a conditional lethal mutant AAV that can grow in cell lines containing an amber suppressor gene.

FIGURE LEGENDS

Figure 1 Structure of the AAV genome and the amber mutation. The AAV2 genome is shown as a single bar with 100 map units scale (1 unit = approximately 47 nucleotides). Stippled boxes indicate terminal repeats (replication origins) and solid circles indicate transcription promoters, (p_5, p_{19} and p_{40}). The polyA site is at map position 96. RNAs from AAV promoters are shown as heavy arrows with the introns indicated by the caret. The coding regions for the four rep proteins (rep78, rep68, rep52 and rep40) and for the viral capsids (VP1, VP2 and VP3) are shown with open boxes. The lower portion shows the extended region of the AAV genome where the amber codon was generated by site-directed mutagenesis.

Figure 2 Replication of the AAV amber mutant in the serine tRNA amber suppressor bearing cell lines. Control cell (SupO) or suppressor cell lines (SupD3 and SupD12) were infected with adenovirus and 1 hour later the different cell lines were transfected with pNTC244 containing the wild type AAV genome (w) or pNTC3 having the amber mutation (a) and incubated at 39.5°C or at 33°C. After appropriate incubation times the replicating viral DNA was isolated and analysed by agarose gel electrophoresis and Southern blotting using AAV2-^{32}P-DNA. II and III indicate non-replicating DNA

forms of input plasmid DNA. RFd, RFm and SS indicate replicating duplex dimer or monomer and single stranded forms respectively, of AAV DNA. M is molecular weight marker of linear duplex AAV2 DNA.

Figure 3 Replication of AAV amber mutant progeny in $tRNA^{ser}$ suppressor bearing cell lines. Viral lysates were prepared after transfecting amber mutant or wild type plasmid (as indicated at the top) into adenovirus-infected cell Sup0, SupD3 and SupD12 cells (designed PO cells and marked 0, 3 and 12 respectively in the figure) and growing at 33°C or 39°C. These lysates were used to infect P1 cultures of Sup0 and SupD12 cells at 33°C. Viral DNA was isolated from the P1 cultures and analysed by agarose gel electrophoresis and Southern blotting with an AAV-^{32}P-probe. The PO cultures were duplicates of those analysed in Figure 2. The letters a through l at the bottom of each track indicate the corresponding cultures in Figures 2 and 3. RFd, RFm and SS indicate the intracellular AAV molecules. M, is molecular weight marker.

Figure 4 Reversion frequency of the amber mutation in pNTC3. Serial dilutions (10°to 10^{-5}) of the viral lysates of the amber AAV mutant (pNTC3 am) and the wild type AAV (pNTC244 wt) were used to infect adenovirus-infection viral DNA replicating forms were analysed. RFd, RFm and SS indicate intracellular AAV DNA species. M indicates a molecular weight marker of duplex AAV DNA.

Figure 5 Production of the rep proteins by the AAV rep amber mutant. Adenovirus-infected Sup0 and SupD12 cells were infected with 10 infectious units of wild type (wt) or amber mutant (am) viral lysates. Rep

protein production was analysed by immunoblotting with an anti-rep antibody as described in Materials and Methods. Ad - adenovirus-infected control cell. Rep78, rep68, rep52 and rep40 indicate AAV2 rep proteins.

Figure 6 Mutagen-induced reversion of the rep amber mutant AAV, NCT3. HelaJW cells (10^6 cells per 35 mm dish) growing at 37°C were infected either with the wild type virus, AAV wt or with the amber mutant virus, AAVam, each at a multiplicity of infection of 10 infectious units per cell as indicated. After two hours at 37°C the virus innoculum was removed and the cells were treated with mutagenic agents (indicated in figure) for 2 hours at 37°C. The mutagen was then removed and cells were infected with the helper adenovirus type 5 at a multiplicity of 3 plaque forming units per cell. After 60 hours at 37°C the cells from each culture were harvested in a final volume of 0.2 ml. This viral lysate was frozen and thawed 3 times and heated at 65°C for 15 min. An aliquot (0.05 ml) of this virus lysate was used to infect 10^6 HelaJW cells (growing at 37°C in a 35 mm dish) together with helper adenovirus type 5 at a multiplicity of 3 plaque forming units per cell. 40 hours later AAV DNA was selectively extracted from the cultures and electrophoresed in a 0.7% agarose gel followed by blotting to a nitrocellulose membrane. AAV DNA was detected by hybridization with an AAV ^{32}P-DNA probe followed by autoradiographic exposure to x-ray film. The mutagenic agents used were MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) at 0.2 μg/ml (tracks 1,5) or 1.1 μg/ml (tracks 2,6); DMBA (7,12-Dimethylbenz[a]-

anthracene) at 0.4 μg/ml (tracks 3,7) or 2 μg/ml (tracks 4,8); HU (hydroxyurea) at 1mM (tracks 11,15); BUdr (5-bromodeoxyuridine) at 0.4 μg/ml (tracks 12,16) or 2 μg/ml (tracks 13,17). Controls included: Ad, lysates from cells infected with adenovirus alone, (track 9); C, lysates from uninfected cells, (track 10); D, lysates from cells infected with AAV wt (track 14) or AAVam (track 18) and treated with dimethylsulfoxide (final concentration 2% v/v)

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a means of preparing conditional-lethal nonsense mutants of AAV by inactivating the rep gene using an amber mutation. The mutant virus was, in this instance, propagated in monkey cell lines expressing an amber suppressor gene. However, the method can be practiced using other cell lines containing the suppressor. The mutant could then be packaged without generation of wild type recombinants directly. Monkey cells containing an inducible amber suppressor tRNA allow propagation and genetic analysis of AAV used herein. However, functional equivalents may be used by the method of the invention

While wild type particles may still be generated by reversion of the amber mutation, such spontaneous reversion occurred at low frequency. The preparations of amber mutants contained less that 1 in 10^5 wild type revertants. Since the particle to infectivity ratio for wild type AAV usually is about 20:1 to 50:1, the amber mutant described here can be used at relatively high multiplicity. The approach is, therefore useful for packaging other AAV mutants and AAV vectors in the

absence of significant wild type recombinants.

Because the spontaneous reversion frequency of the mutant virus is very low the invention can be used as an assay for effects of mutagenic agents in cultured human cells. Cultured human cells do not have naturally occurring amber suppressor genes and thus the mutant AAV cannot grow in such cells even when coinfected with a helper adenovirus. However any mutagenic agent which causes the amber mutation in the infecting AAV genome to revert to wild type or pseudo-wild type should allow expression of a functional AAV rep gene and thus the revertant virus would amplify and propagate. This provides a simple, novel and sensitive assay for analysis and detection of mutagenic agents by screening in cultured human cells. It is demonstrated that the reversion frequency of the mutant virus can be greatly increased by several orders of magnitude in a single growth cycle by known mutagenic agents. This provides a rapid and sensitive assay for mutagenic and potential genotoxic effects of compounds in human cells. Because AAV can grow in any type of human cells when infected with a helper adenovirus this mutagen assay will be applicable to any human cell lines.

Cells

Human Hela cells (HelaJW cells) were originally obtained from J. Janik (NIH) at passage 35 and were grown in monolayer cultures in Eagle's minimal essential medium supplemented with 10% fetal calf serum in 5% CO_2.

The three monkey cell lines, BSCSupO, BSCSupD3 and BSCSupD12 (Sedivy et al., Cell, 1987, 50:379) were obtained at passage 3 from J. Sedivy and P. Sharp

(MIT). All three lines designated SupO, SupD3 and SupD12, respectively, were grown at 39.5°C in Dulbecco's modified Eagle's medium with 10% fetal calf serum in 5% CO_2 in 10 cm dishes. For routine passage the cells were split 1:50 about every six days. The antibiotic geneticin (700 μ/ml total concentration) was included in the medium in alternate passages and care was taken to avoid exposure of the cell cultures to temperatures below 39.5°C.

For experiments, one 10 cm dish of confluent cells was split 1:4 into 6 cm or 1:12 into 3.5 cm dishes. The cultures were immediately placed at 33°C and the medium was changed after 24h. After an additional 24 h at 33°C the cells were infected with virus or transfected with DNA. Under these conditions, at the time of infection or transfection, the dishes with SupD3 or SupD12 cells contained about half the number of cells as the SupO cultures. This reflected induction of the suppressor tRNA in SupD3 and SupD12. Parallel control cultures were maintained at 39.5°C. After 24 h at 39.5°C these cultures were refed with fresh (prewarmed) medium, then infected with virus or transfected with DNA 4 h later.

In more recent experiments for propagation of the AAV<u>am</u> mutant, NTC3, the SupD12 cells are plated at 1:4 or 1:12 splits as above but grown overnight at 39.5°C or 72 h after infection or transfection at 33°C. The next day the cells are shifted to 33°C and 2 hr later infected with adenovirus and AAVam virus or transfected with pNTC3 DNA.

For analysis of AAV DNA replication, 39.5°C cultures were harvested at 24 h after virus infection

or 40 h after DNA transfection and 33°C cultures were harvested at 40 h after infection or 70 h after transfection. For assay of viral proteins or infectious progeny virus, cells were harvested at 48 h after infection or transfection at 39.5°C. Viral lysates from transfected cultures were concentrated ten fold. The choice of harvesting times reflects the two-fold decrease in the AAV growth rate at 33°C (Myers et al., J. Virol., 1980, 35:65) and the less efficient growth in transfected cultures (Laughlin et al., Gene, 1983, 23:65; Tratschin et al., J. Virol., 1984, 51:619).

Human HeLa cells were grown at 37°C in Eagle's minimal essential medium with 10% fetal calf serum.

DNA transfection

Cells were plated as described above and transfected with the appropriate plasmid according to the $CaPO_4$ precipitation procedure (Tratschin et al., Mol. Cell. Biol., 1984, 4:2072). When required for AAV replication, the cells were infected with adenovirus type 5 at a multiplicity of infection of 3 to 5 infectious units per cell one hour prior to DNA transfection.

Virus growth and infectivity assay.

Adeno-associated virus (AAV) type 2 and the human adenovirus type 5 (Ad) were grown as described (Carter et al., Virology, 1979, 92:449). Infectious titers of AAV were determined in the nuclear fluorescent-focus assay with indirect immunofluorescence using anti-AAV capsid rabbit antiserum and FITC-conjugated goat anti-rabbit IgG (Carter et al., Virology, 1979, 92:449).

Plasmids

Stocks of plasmids were grown in E. coli and purified by standard procedures (Maniatis et al., "Molecular Cloning" 1982, Laughlin et al., Gene, 1983, 23:65; Tratschin et al., J. Virol., 1984, 51:619). All plasmid preparations used for transfection or DNA sequencing were twice banded in CsCl. The plasmid pNTC244 contains a wild type AAV2 genome inserted in the polylinker sequence of the phagemid pTZ19U (Chejanovsky and Carter, Virology, 1989, 173:120).

Construction of mutants

The mutant pNTC3 was constructed by using the synthetic oligonucleotide 5'-GATTACCTAGGAGAAGCAG-3' to introduce an amber codon into the AAV rep gene in pNTC244 as described further below. The oligonucleotide was produced on an Associate Biosystem Model 381A synthesizer using B-cyanophosphoramidite chemistry and was subsequently purified by gel electrophoresis. The oligonucleotide was then constructed into the pNTC244 background according to the general procedures of Zoller and Smith Methods in Enzymology, 1983, 100:468 and Kunkel Proc. Natl. Acad. Sci. USA., 1985, 82:448 as implemented in the BioRad Muta-gene system (BioRad Laboratories, Richmond, CA). Briefly, the synthetic oligonucleotide was used to prime a DNA polymerase reaction with a single-stranded template consisting of an M13 vector containing an insert of a sub-genomic portion (nucleotides 449 to 1045) of the AAV genome. Plaques containing the mutations were selected and the successful generation of the nutation was checked by DNA sequencing. This mutant region was then reconstructed into a complete

AAV genome in the plasmid pNTC244 to generate the mutant pNTC3. The presence of the appropriate mutation in the final AAV-plasmid recombinant, pNTC3 was again determined by DNA sequencing. Large scale amounts of the plasmid were then prepared for transfection assays.

Sequencing was performed using the dideoxynucleotide procedure, [^{35}S]-labeled nucleotides, and the mutant T7 DNA polymerase, sequenase (United States Biochemical Corporation) on either single-stranded phagemid or double-stranded plasmid templates.

Analysis of viral DNA replication.

AAV DNA replication was analyzed using the modified Hirt SDS-high salt lysis procedure (Carter et al., Viroloy, 1983, 126:505) to extract viral-specific DNA. This DNA was electrophoresed in 0.7% agarose gels (11 x 14 cm) for 16 hr at 10 mA/gel and blotted to nitrocellulose followed by hybridization with AAV ^{32}P-DNA labeled in vitro by random priming (Feinberg and Vogelstein, Anal. Biochem., 1982, 132:6).

Analysis of AAV protein synthesis.

Synthesis of AAV rep proteins (Rep78, Rep68, Rep52 and Rep40) was determined using specific antisera in immunoblotting assays (Medelson et al., J. Virol., 1986, 60:823) using a rabbit antiserum (anti rep78.93) raised against a 93% region of rep78 expressed from a prokaryotic vector in E. coli (Trempe et al., Virology, 1987, 161:18)

Assay of reversion induced by mutagenic agents.

The ability of mutagenic agents to induce reversion of the amber mutation in NTC3 to wild type or pseudo-wild type was tested using HeLaJW cells. A stock of AAVam particles was prepared by transfection of pNTC3

into SupD12 cells in the presence of the helper adenovirus and growth at 33°C as described above. The infectivity titer of the AAVam stock was determined as described above.

To determine the effect of mutagens HeLaJW cells (5 x10^5 cells per 35 mm dish grown at 37°C) were infected with the AAVam stock at a multiplicity of 10 infectious units per cell. The virus innoculum was allowed to absorb in a volume of 2 ml of complete medium (Eagle's minimal essential medium with 10% fetal calf serum and antibiotics) at 37°C for 2 hr. The innoculum was then removed, the cell monolayer was washed twice with 5 ml of complete medium then 2 ml of complete medium was added to the dish. The mutagenic agent was then added to the medium and the cells incubated for 2 hr at 37°C. The mutagenic agents used were: MNNG (N-methyl-N'-nitrosoguanidine) obtained from Sigma Chemical Company, St. Louis, MO; DMBA (7,12-dimethylbenz[a]-anthracene) obtained from Aldrich Chemical Company, Milwaukee, WI; BUdR (5-bromodeoxyuridine) and HU (hydroxyurea)both obtained from Calbiochem. MNNG and DMBA were prepared as stock solution at 400 μg/ml in DMSO (dimethylsulfoxide) and added to the cell medium to give final concentration of 0.22 μg/ml or 1:1 μg/ml (MNNG) or 0.4 μg/ml and 2 μg/ml DMBA. Controls included cells treated with DMSO alone at final a concentration of 2% (v/v). BUdR was made as an aqueous stock solution at 400 μg/ml and added to 0.4 μg/ml or 2 μg/ml final concentration. HU was made as an aqueous solution at 100 mM and added to a final concentration of 1 mM.

After incubation with the mutagens, the medium was removed, the cell monolayer was washed twice with medium and finally fresh medium (2 ml) was added to the dish. Adenovirus type 5 was then added to the cells at a multiplicity of 3 plaque forming units per cell and the cells were incubated at 37°C for 60 hr. The cells were then harvested from the dish, washed with 5 ml of phosphate buffered saline (PBS) then washed with 2 ml of medium and finally resuspended in 0.2 ml PBS. The cells were then frozen and thawed three times and heated at 65°C for 15 min. The debris was then removed by centrifuging and the supernatant was taken as the viral lysate. An aliquot (0.05 ml) of each lysate was used to infect fresh cultures of HeLaJW cells (5×10^5 cells per 35 mm dish) in the presence of adenovirus type 5 (3 plaque forming units per cell). 40 hours after infection AAV DNA was selectively extracted and analyzed by gel electrophoresis, blotting, hybridization with an AAV ^{32}P-DNA probe and autoradiography as described above. The agarose gel contained ethidium bromide to allow visualization of the DNA under UV light prior to blotting. The viral DNA from AAV wt infections and the high levels of revertants of AAVam induced by MNNG or DMBA could be directly seen in the ethidium bromide stained gel prior to blotting (data not shown).

RESULTS

AAV genome structure and construction of a Rep amber mutant.

The structure of the AAV2 genome is shown in Fig. 1 (Srivastava et al., 1983). In the right half of the genome a large open reading frame (ORF2) is expressed

from RNA transcribed from the p_{40} promoter and codes for three overlapping capsid polypeptides, VP1, VP2 and VP3 (Bacerra et al., J. Virol., 1988, 82:7919; Cassinnotti et al., Virology, 1988, 167:176; Trempe and Carter, Virology, 1988, 62:3356). In the left half of the genome, a large open frame ORF1 yields four overlapping rep proteins (Mendelson et al., J. Virol., 1986, 60:823). Thus, ORF1 comprises the rep gene (Hermonat et al., J. Virol., 1984, 51:329; Tratschin et al., J. Virol., 1984, 51:611) which may consist of the two overlapping genes. P_5 rep is transcribed from the p_5 promoter and yields two proteins, Rep78 and Rep68. P_{19} rep is transcribed from the p_{19} promoter and yields two proteins Rep52 and Rep40. The rep proteins are required for AAV DNA replication and also mediate positive and negative trans-acting pleiotropic effects on genes expressed from AAV or heterologous viral and cellular promoters (Tratschin et al., Mol. Cell. Biol., 1986, 6:2884; Labow et al., J. Virol., 1986, 60:251 and Mol. Cell. Biol., 1989, 7:1320; Mendelson et al., Virology, 1988, 166:612; Trempe and Carter, J. Virol., 1988, 62:68).

A nonsense mutation was introduced into ORF1 immediately upstream of the Bam-H1 site at map position 22 by converting a serine codon to the amber codon and reconstructing this into the wild type plasmid pNTC244 to yield the mutant pNTC3 (Fig. 1). If the amber mutation resulted in efficient translation termination it was expected to truncate all four rep proteins and yield a mutant that was rep. This mutant should be suppressed by the amber suppressor tRNA present in SupD3 and SupD12 cells. Since AAV2, like adenovirus

and SV40, does not use the amber codon as a terminator for any genes it was not expected that the amber suppressor would interfere with normal growth of AAV.

Growth of AAV on monkey cells.

Monkey cell lines show varying degrees of permissiveness for replication of human adenovirus (Rabson et al., Proc. Soc. Exptl., Biol. Med., 1964, 116:187; Rice and Klessig, J. Virol., 1984, 49:35) due to inefficient expression of adenovirus late proteins. Monkey cells express a similar host range restriction for expression of AAV2 capsid proteins when using a human adenovirus as helper (Buller et al., J. Gen. Virol., 1979, 40:241). For both AAV and adenovirus this host range restriction may be alleviated by coinfection with SV40 (Rabson et al., 1964; Buller et al., 1979). The host range helper function is mediated by SV40 T-antigen and requires only the carboxyl-terminal region of the T-antigen. Thus, a mutant SV40, tsA58, containing a temperature sensitive lesion (Tegtmeyer and Ozer, J. Virol., 1971, 8:516) expresses a T-antigen which does not function for SV40 DNA replication but still provides the host-range helper function for adenovirus.

All three cell lines Sup0, SupD3 and SupD12 contain the SV40 tsA58 gene so should be permissive for AAV2 using the human adenovirus 5 as a helper. As shown in Table I, all three cell lines supported AAV growth at both 33°C and 39.5°C. The lower yield at 33°C probably reflects the slower growth rate at this temperature.

SupD2 and SupD12 differ from Sup0 in having also an integrated human amber suppressor $tRNA^{ser}$ contained in an SV40 replicon (Sedivy et al., Cell, 1987, 50:379).

Upon downshift of cells from 39.5°C to 33°C, the T-antigen expressed by the tsA58 gene becomes functional for SV40 DNA replication. This leads to amplification of the SV40 replicon containing the amber suppressor and provides a greatly increased copy number and high expression of the suppressor tRNA which in turn allows efficient amber suppression. Thus, the data in Table I also show that expression of the amber suppressor was not inhibitory for growth of AAV.

Suppression of the AAV rep gene nonsense mutation in SupD3 and SupD12 cells.

Results of examination of suppression of the nonsense mutation in AAV2 by transfection of the pNTC3 or pNTC244 DNA into adenovirus-infected cells and subsequent analysis of the intracellular AAV DNA by gel electrophoresis can be seen in Figure 2. At 39.5°C or 33.0°C, the wild-type plasmid, pNTC244, showed AAV intracellular DNA species expected for normal AAV DNA replication. The wild-type plasmid replicated less well at 33°C than at 39.5°C which may reflect the slower growth rate of the cells at the lower temperature. The mutant pNTC3 did not replicate in any of the cell lines at 39.5°C but the open circle (form II) or linearized (form III) species of the input plasmid DNA were seen. At 33°C, replication of the mutant AAV genome from pNTC3 was readily detected in SupD12 cells and faintly on SupD3 cells but not in Sup0 cells. Thus, the amber mutation in the AAV rep gene of pNTC3 was only suppressed on SupD12 or SupD3 at 33°C. The SupD12 cells apparently suppressed the amber mutation more efficiently than did SupD3 cells (Fig. 2). This is consistent with evidence from Sedivy et al

(1987) that, for a CAT gene containing an amber mutation, the level of suppression was 50 to 70% in SupD12 and about 20 to 30% in SupD3.

Suppression of the nonsense mutation in pNTC3 yielded viral particles containing mutant AAV genomes.

It was conceivable that the replication of pNTC3 in SupD3 or SupD12 cells at 33°C simply reflected a high frequency of reversion of the nonsense mutation in these cells rather than suppression by the induced suppressor $tRNA^{ser}$. To test this, a set of cell culture (PO cultures) which were duplicates of those described in Figure 2, were harvested for preparation of virus lysates. Aliquots of these lysates were then used to infect Sup0 or SupD12 cells (P1 cultures) in the presence of adenovirus at 33°C. Viral DNA was extracted and analysed by agarose gel electrophoresis as shown in Fig 3. The presence of replicating AAV DNA in the P1 cultures is diagnostic of infectious AAV particles in the PO lysates.

As can be seen in Fig 3, the pNTC244 wild-type virus grown in PO cultures of all three cell lines yielded infectious AAV that replicated P1 cultures of both Sup0 and SupD12 and was not dependent upon amber suppression. In contrast, the PO cultures of SupD3 or SupD12 transfected with pNTC3 yielded virus that replicated in P1 cultures of SupD12 cells but not in Sup0 cells. The PO cultures of Sup0 cells transfected with pNTC3 did not yield any infectious virus.

The results in Figure 3 are consistent with those of Figure 2. Together they show that the bulk of the infectious AAV virus produced by transfection of SupD3 or SupD12 cells with pNTC3 retained its amber mutant

phenotype.

In the experiment of Fig 3, comparison of the relative amounts of RF DNA suggested that the yield of infectious mutant virus from the PO SupD3 cells was less than from the SupD12 cells. This is consistent also with the less efficient amber suppression on SupD3 cells observed in Fig 2. For subsequent experiments we used only the SupD12 cells as the permissive host for pNTC3.

Efficiency of suppression of pNTC3 on SupD12 cells.

To measure the relative efficiency of amber suppression, pNTC244 and pNTC3 were grown on both SupD12 and Sup0 cells at 33°C, then assayed for infectivity of the resulting virus stocks on SupD12 cells at 33°C. The plasmid pNTC244 gave an efficient yield of virus when grown on either cell line. pNTC3 yielded no detectable infectivity when transfected on Sup0 cells but on SupD12 cells at 33°C gave a yield about 1/10 that of wild-type. This shows that the apparent efficiency of amber suppression was about 10% in SupD12 cells and is consistent with the DNA replication analysis of Figure 2. The level of amber suppression was lower than the 50 to 70% suppression reported for an amber mutation in a CAT gene in SupD12 cells (Sedivy et al., Cell, 1987, 50:379). However, for a virus such as AAV with a complex growth cycle, the overall level of suppression may reflect cascade effects such as a decreased level of rep protein leading to a further decrease in replication and in the number of single-strands available for encapsidation.

Reversion of the amber mutation in pNTC3.

The experiment of Fig 3 showed that most of the virus particles generated by growth of pNTC3 on the amber suppressor cell lines (SupD3 and SupD12) at 33°C exhibited an amber mutant phenotype. However, this did not readily determine the level of reversion of the amber mutation.

Reversion of the mutation was examined in two ways. Preparations of pNTC3 were grown in permissive conditions in SupD12 cells at 33°C then examined for AAV infectivity under non-permissive conditions in Sup0 cells at 33°C. Under these conditions only wild-type revertants should score as infectious virus. Infectivity was measured using either the immunofluorescence assay (Carter et al., Virology, 1979, 92:449) or a DNA replication assay.

The immunofluorescence assay (Table 2) performed on Sup0 cells at 33°C showed a few cell nuclei that were positive for AAV capsid fluorescence when stocks of pNTC3 grown on SupD12 at 33°C were examined. Thus, some wild-type revertants apparently existed in the virus population. In contrast, no infectious virus were detected in lysates of Sup0 cells infected with pNTC3 (Table 2). However the immunofluorescence assay for AAV infectivity relies on the ability to detect the level at which 63% of the maximum number of nuclei are fluorescent for AAV capsid antigen. Thus, the actual titer of the revertants could not be determined readily because the number of fluorescent nuclei was too low and only the extreme end point of the titration was detected. Nevertheless, the titer of the pNTC3 derived virus grown on SupD12 cells was at least five orders of

magnitude lower than that of the virus derived from pNTC244 (Table 2). This showed that the reversion frequency in pNTC3 stocks grown on SupD12 cells at 33°C was less than 10^{-5} and probably less than 10^{-6}.

As an alternate measure of reversion, a DNA replication assay was used (Figure 4). In this assay, Sup0 cells were infected with serial dilutions of virus stocks and viral DNA replication was measured. The level of RF DNA replication thus reflected the level of infecting wild-type genomes and depended upon the presence of a functional rep gene.

A stock of wild-type AAV, originally grown from pNTC244 in SupD12 cells, showed a similar infectivity titration in either SupD12 or Sup0 cells at 33°C (Fig. 4). A stock of mutant virus grown in parallel from pNTC3 had about 10 fold less infectivity than the wild-type stock in SupD12 cells. This is consistent with the data above (Table 2) that the yield of mutant virus was only about 10% of that of wild-type when grown in SupD12 cells. Prolonged autoradiographic exposure of the gel in Fig. 4 (not shown) also detected replication of pNTC3 virus in SupD12 cells in the 10^{-5} dilution and an even fainter signal only in the 10^{0} dilution of pNTC3 in the Sup0 cells. Thus, level of reversion is less than 10^{-5} and probably below 10^{-6}. This is consistent with the data in Table 2.

<u>Suppression of the amber mutant in SupD12 cells Yields synthesis of rep protein</u>.

Suppression of the amber mutation in pNTC3 presumably resulted in synthesis of rep proteins at the permissive temperature in SupD12 and SupD3 cells. Rep proteins could not be easily detected in adenovirus-

infected SupD12 or SupD3 cells after transfection at 33•C with pNTC3 although they could be detected after transfection with pNTC244 (data not shown). This reflected much lower expression of rep proteins after transfection with AAV plasmids than after AAV particle-mediated infections. Also, the efficiency of suppression of the mutation in pNTC3 was only about 10% when grown on SupD12 cells.

To demonstrate that amber suppression did allow synthesis of rep protein from the amber mutant genome, the experiment shown in Figure 5 was performed. Virus stocks containing wild-type or amber mutant genomes were generated from pNTC244 or pNTC3 respectively in SupD12 cells and used to infect SupD12 or Sup0 cells at 33°C. Wild-type AAV expressed similar amounts of the rep proteins in either cell line. The amber mutant expressed about 10 fold less amounts of both major rep proteins, rep78 and rep52 in SupD12 cells but not in Sup0 cells. These results showed that the amber suppressor did allow expression of rep proteins but at a lower overall efficiency than for wild type. Thus, the less efficient DNA replication and infectious virus production by the amber mutant was reflected in a lower level of rep protein synthesis.

Use of amber mutant AAV to generate AAV transducing vectors.

Identification of rep proteins and genetic analysis using mutant AAV plasmids has shown that rep is required for DNA replication in DNA-mediated infections. Furthermore, the accumulated evidence indicates that rep is required in normal AAV particle-mediated infections. The level of duplex RF

replication and the yield of infectious virus for the amber mutant appears to reflect the level of suppression of the rep mutation and thus reflects the level of rep protein.

Using procedures analogous to those described herein for the rep gene, we have also constructed an amber mutation in the cap gene of AAV to block synthesis of the capsid proteins VP1, VP2, VP3 (J. Smuda, N. Chejanovsky, B.J. Carter, manuscript in preparation). The cap mutation can also be suppressed efficiently as for the rep gene to provide capsid proteins without generating a wild type cap gene. Thus, both classes of suppressible mutant genes may be used to supply AAV rep and/or cap gene functions under conditions in which infectious wild type AAV is not generated. These mutant rep or cap genes can be supplied as part of an AAV vector by a cotransfecting DNA molecule or as part of the infecting helper adenovirus or by stable maintenance of these genes in cell lines containing an inducible suppressor. These mutant genes can allow replication and packaging of AAV vectors (not containing a functional rep or cap gene) into AAV particles, under conditions where recombination does not yield wild type infectious AAV or wild type functional rep or cap genes, to produce high levels of AAV particles containing the vector.

Vectors containing foreign genes or cDNA may be packaged into AAV particles by the method of the invention. Such vectors produced by the inventive techniques may be used to infect suitable cell cultures to produce high levels of proteins such as hormones, growth factors, enzymes or pharmacologically useful

proteins. Furthermore, AAV vectors produced by the inventive means may be used to introduce genes or parts of genes into cells or patients for the purposes of diagnosing, alleviating or correcting genetic diseases or other diseases.

Use of mutant virus to assay mutagenesis in cultured human cells.

The inventive procedure produces pure populations of mutant virus which may be used as the basis of an assay for mutagenesis in cultured human cells and thus for screening potential mutagenic and carcinogenic agents. Such agents may be tested by their effect on the reversion frequency of the mutant virus.

The use of the invention to detect mutagenesis in cultured human cells is demonstrated in the experiment shown in Figure 6. In this experiment human HeLaJW cells were first infected with the AAVam virus stocks then treated with various mutagenic agents. Helper adenovirus was then added to the cells and stocks of progeny AAV were harvested 60 hr later. These AAV stocks were then used to infect fresh HeLaJW cells in the presence of adenovirus (But in the absence of any mutagen) and AAV DNA synthesis was measured. HeLaJW cells do not contain an amber suppressor. In these cells AAVam can only generate progeny AAV if reversion of the amber mutation occurs.

In the experiment of Fig. 6 the negative controls of uninfected cells (track 10) or cells infected only with adenovirus (track 9) yielded no progeny virus. Similarly in the positive controls (tracks 1-4, 11-14) HeLaJW cells infected with AAV wt yielded large amounts of progeny AAV in the presence (tracks 1-4, 11-13) or

absence (track 14) of mutagen treatment.

In Figure 6 control cells infected with AAVam and treated only with dimethylsulfoxide (not known to be a mutagen) yielded not detectable progeny virus. Thus spontaneous reversion (as noted above) was extremely low. When the AAVam infected cells were treated with the mutagen MNNG (tracks 5,6) or DMBA (tracks 7,8) very high levels of progeny AAV were generated and the levels of AAV DNA replication approached those of the wild type virus. Thus both mutagens induced reversion to wild type or pseudo-wild type or both. Hydroxyurea (track 15) also induced reversion of AAVam but at a lower level. Also with BUdR (tracks 16,17) there was a low but detectable production of progeny revertant virus which was more readily seen in longer autoradiographic exposures (not shown).

The invention of the mutant AAV provides a novel assay for screening mutagenicity of compounds in human (or other mammalian) cells. Various assays for mutagenesis have been proposed previously for use in mammalian cells but many of these use shuttle vectors to measure the "forward" mutation rate of an active gene from a wild type to a mutant phenotype. However, the spontaneous rate of such mutations is frequently prohibitively high to readily measure the specific effect of mutagens in inducing such forward mutations.

Mutagenicity of any agent may be evaluated by any method which provides for growth of a cell line which has been infected with a virus containing an amber mutation in an essential AAV gene in the presence of a putative genotoxic agent. The mutagenicity of the putative genotoxic agent is evidenced by increased

incidence of wild type or pseudo wild type virus, as measured by viral DNA or proteins.

It was pointed out recently (Greenspan et al., Mol. Cell. Biol., 8:4185-4189) that analysis of the effect of mutagens on reversion to wild type is much more sensitive because the spontaneous rate of reversion is much lower than the forward rate. The invention described here has this advantage and several other potential advantages. First, the mutant virus can be infected into cells of any human or other mammalian tissue eg. liver, lung. Second, wild type (and thus revertant) AAV will grow in any human cells from any tissue provided a helper adenovirus is present (Carter, Handbook of Parvoviruses Vol. 1, CRC Press, 1989 pp 255-282). Thus the invention will be useful for analysing mutagenic effects of compounds whose mutagenic or genotoxic effect is actually mediated by an intermediate metabolite which is generated by tissue specific enzymes. In contrast, most shuttle vectors previously designed for mutagenesis assays in mammalian cells are restricted in their replication to specific types of cells or even specific host. Third, in a normal AAV growth cycle a single infectious unit generates from one infected cell a burst of up to 10^4 infectious units which represents up to 10^6 AAV genomes (Carter et al., Virology 1979, 92:449). Thus one mutagenic event per 10^6 cells treated with mutagen can yield 10^4 infectious units. When 25% of this lysate was used to infect a second culture of 10^6 HeLa cells (as in the experiment of Figure 6) a similar amplification occurs. Thus one mutagenic event can give 10^{10} AAV genomes in the second culture. This represents an

enormous amplification of the signal from the initial mutagenic event thus increasing the sensitivity of the assay. It also has practical advantages since this large number of genomes is amendable to rapid assay using automated procedures and various types of nucleic acid hybridization assay or various immunological assays for either AAV rep protein or AAV capsid proteins. Such rep and capsid proteins are clearly defined and specific antibodies have been developed (Mendelson et al., J. Virology 1986, 60:823; Trempe et al., Virology 1987, 161:18; Carter et al., Virology 1979, 92:449).

A fourth novel feature of the present invention is that the AAV genome in the infecting particle is single-stranded DNA. All other shuttle vectors or mammalian viruses suggested for mutagenesis assays have double stranded DNA genomes. Because the infecting AAVam genome is single-stranded (prior to conversion to the first double strand intermediate by host cell enzymes) it offers a unique type of target which may detect additional mutagenic events more readily than with double stranded genomes.

Finally, several extensions of the present invention are now obvious. As noted above other types of human or mammalian cells can be used for mutagen testing. Secondly, other types of mutations can be introduced into the AAV genome, such as nonsense mutations at other serine or non-serine codons or in non-essential regions of the rep or capsid protein. This would allow design of the system to detect certain classes of reversion such as transitions or transversions. Also, introduction of additional

nucleotides or deletion of one or two nucleotides would allow testing for compounds that cause frameshift mutations. Also, location of mutations within non-essential regions of rep or capsid genes would permit detection of reversion due to deletions. Generation of cell lines containing the AAVam genome integrated into the cellular chromosome would provide a highly sensitive assay for reversion because treatment of such cells with mutagens leading to reversion of the am mutation would render the integrated AAV genome rescuable and amplifiable upon superinfection with a helper virus (eg. adenovirus).

E. coli. containing the plasmid pNTC3 has been deposited in the American Type Culture Collection in Rockville, Maryland under the provisions of the Budapest Treaty and will be maintained for at least 30 years following issuance of the patent. The accession number is 68000 and was received on June 1, 1989.

Table I

Growth of AAV2 on monkey cell lines[a]

Cell line	AVV2 titer at[b]: 33°C	39.5°C
BSC SupO	4.8×10^8	7.2×10^9
BSC SupD3	4.8×10^8	3.2×10^9
BSC SupD12	5.6×10^8	5.6×10^9

[a]. Cells were plated in 60 mm dishes at approximately 10^6 cells per dish and grown at 39.5°C or 33.0°C then infected with AAV2 (1 i.u./cell) and Ad5 (5 i.u./cell). For cultures placed at 39.5°C, cells were infected after 24 hr and lysates for AAV assay were prepared at 48 hr after infection. For cultures placed at 33 C, cells were infected with AAV2 and Ad5 after 48 hr and virus assay lysates were prepared at 72 hr after infection.

[b]. Lysates were frozen and thawed six times, heated at 60°C for 20 min and clarified by low speed centrifugation. The AAV virus titer was determined using the fluorescent focus assay (Carter et al., Virology, 1979, 92:449) on Hela cells.

Table 2

Infectious titer of wild type and mutant AAV stocks

Virus stock[a]	Grown on:	AAV2 infectivity assayed in[b] SupD12	Sup0
AAV2 (pNTC244)	SupD12	4.0×10^8	1.5×10^8
AAV2 (pNTC244)	Sup0	3.2×10^8	1.4×10^8
AAV2 (pNTC3)	SupD12	4.0×10^7	$<1.0 \times 10^3$
AAV2 (pNTC3)	Sup0	0	0

[a] Virus stocks were prepared by transfecting 1 μg of the indicated plasmid onto SupD12 or Sup0 cell at 33°C. Lysates were prepared at 90 hr after transfection

[b] Infectivity of the viral stocks was assayed at 33 C in SupD12 or Sup0 cells using the indirect immunofluorescence procedure (Carter et al., Virology, 1979, 92:449). Titers are expressed as AAV2 infectious units per ml.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 29 line 13 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

American Type Culture Collection

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [5]	Accession Number [6]
June 1, 1989	68000

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

Claims:

1. A method of evaluating mutagenicity of a putative genotoxic agent comprising the steps of:

(1) infecting a cell culture with a virus containing an amber mutation within the genome;

(2) exposing the infected cell culture produced in step (1) to a putative genotoxic agent; and

(3) evaluating the cell culture produced in step (2) to determine extent of reversion of virus in the culture to wild type or pseudo wild type by identification and quantitation of viral DNA or protein produced by revertent replicating virus.

2. A procedure of claim 1 used to screen genotoxic agents or potential genotoxic agents for an effect on reversion frequency of the mutant virus to determine possibility of mutagenic, carcinogenic or genotoxic potential.

3. A comparison of matter comprising a culture of a cell line which has been infected with a virus containing an amber mutation in an essential AAV gene and a putative mutagenic agent.

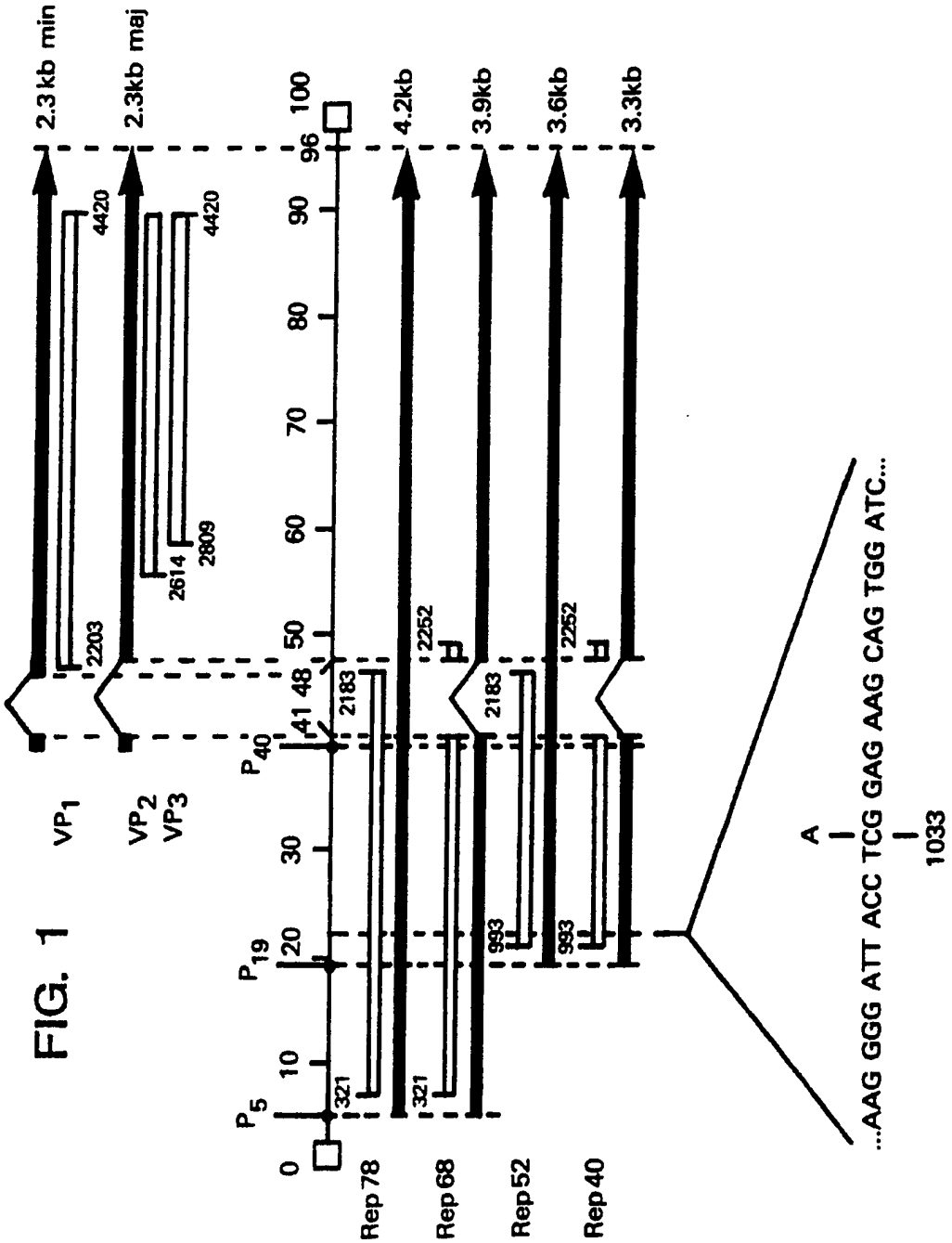

2.3 kb min
2.3kb maj
4.2kb
3.9kb
3.6kb
3.3kb
VP1
VP2
VP3
P5
P19
P40
Rep 78
Rep 68
Rep 52
Rep 40
...AAG GGG ATT ACC TCG GAG AAG CAG TGG ATC...
A
1033

FIG. 1

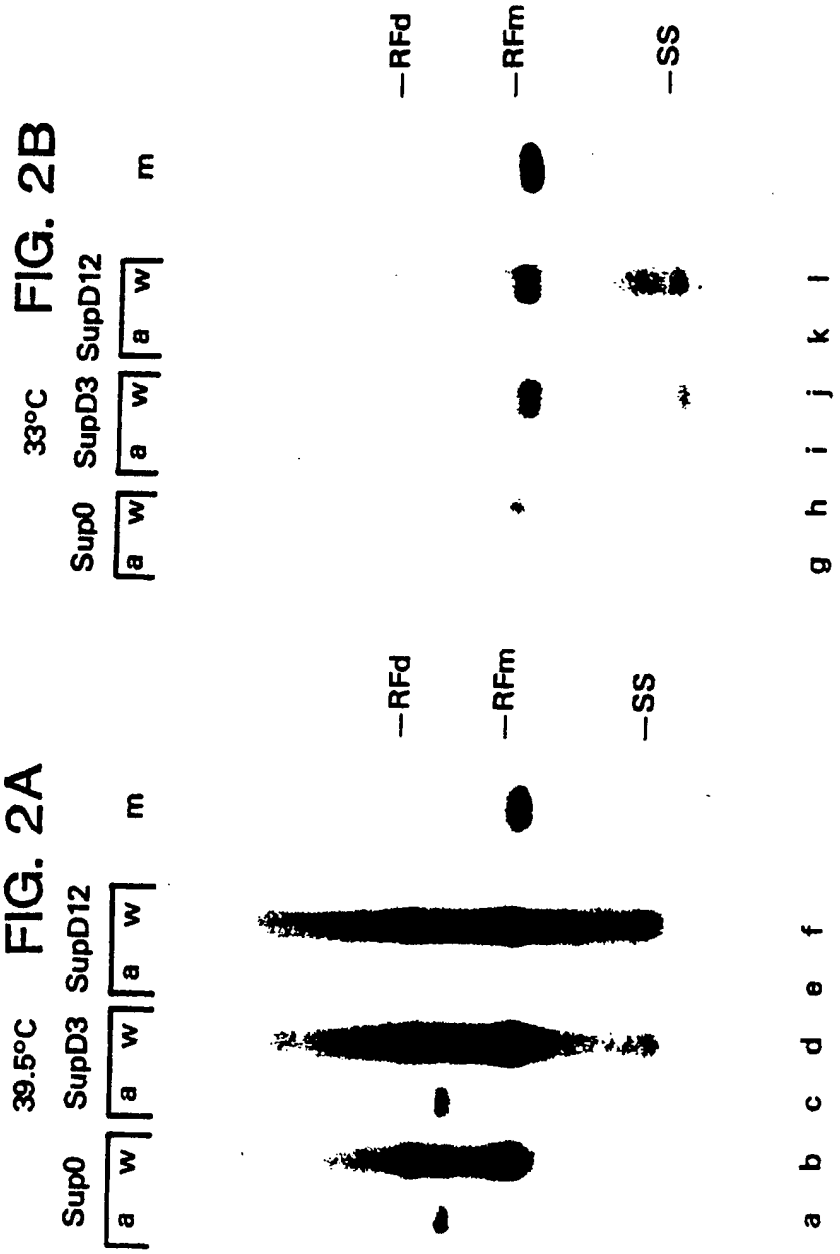


FIG. 2B
33°C
Sup0
SupD3
SupD12
a w a w a w
m
—RFd
—RFm
—SS
g h i j k l
FIG. 2A
39.5°C
Sup0
SupD3
SupD12
a w a w a w
m
—RFd
—RFm
—SS
a b c d e f

FIG. 3

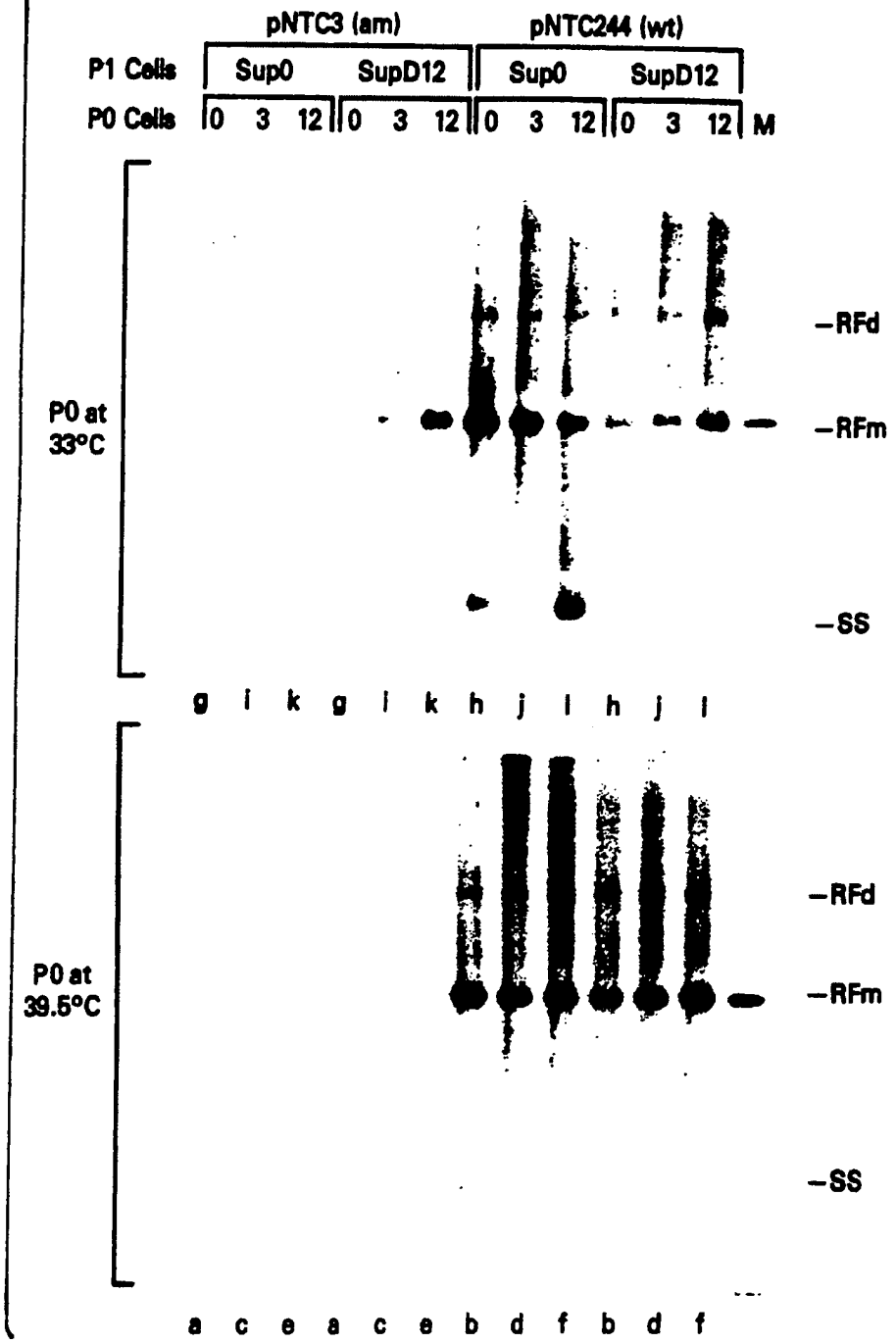

pNTC3 (am)
pNTC244 (wt)
P1 Cells
Sup0
SupD12
Sup0
SupD12
P0 Cells
0 3 12 0 3 12 0 3 12 0 3 12 M
P0 at 33°C
–RFd
–RFm
–SS
g i k g l k h j l h j l
P0 at 39.5°C
–RFd
–RFm
–SS
a c e a c e b d f b d f

FIG.4

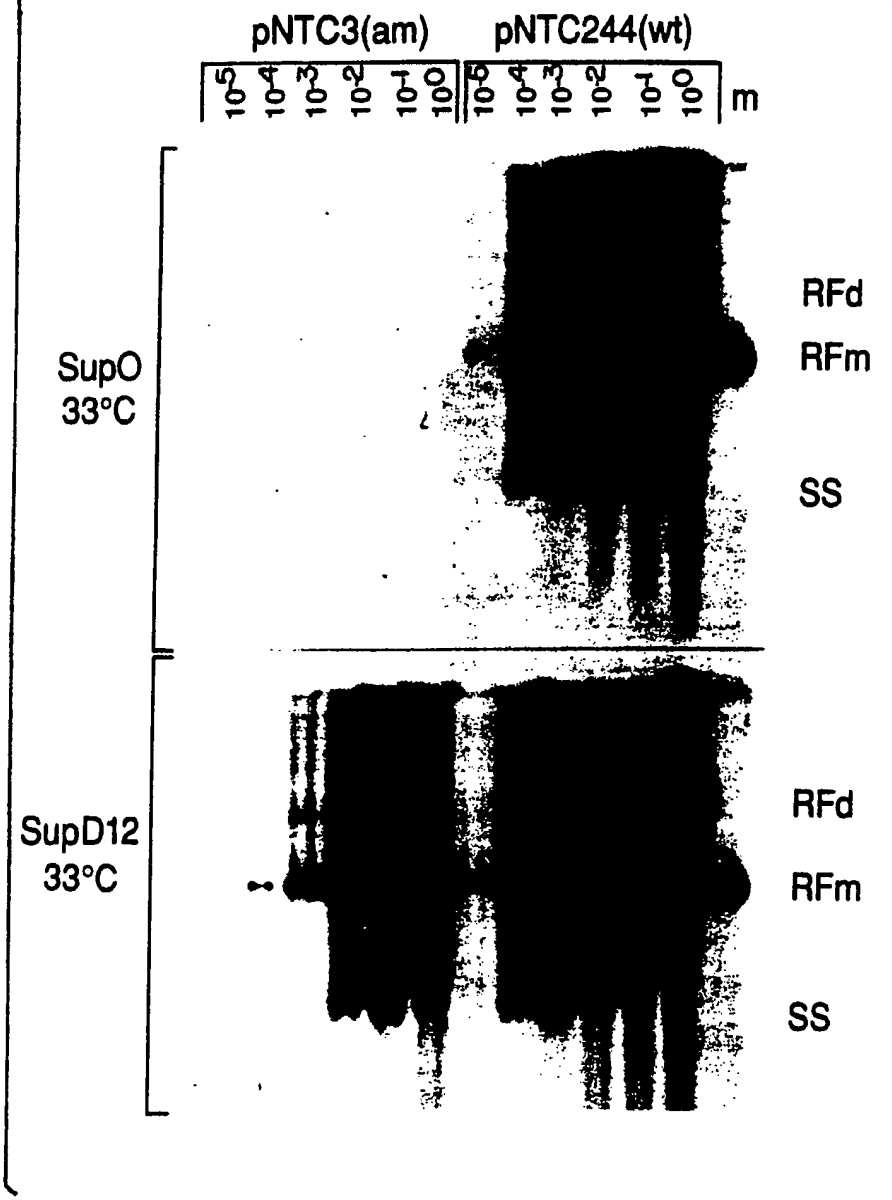
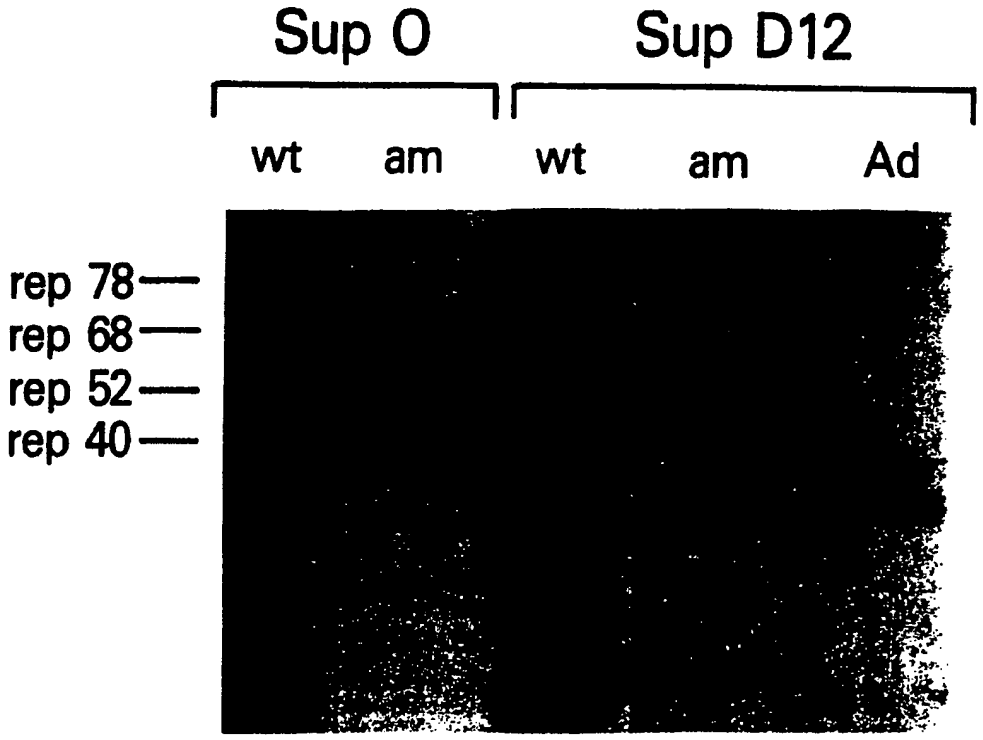

pNTC3(am)
pNTC244(wt)
5 4 3 2 1 0 5 4 3 2 1 0
10 10 10 10 10 10 10 10 10 10 10 10
m
SupO
33°C
RFd
RFm
SS
SupD12
33°C
RFd
RFm
SS

FIG. 5



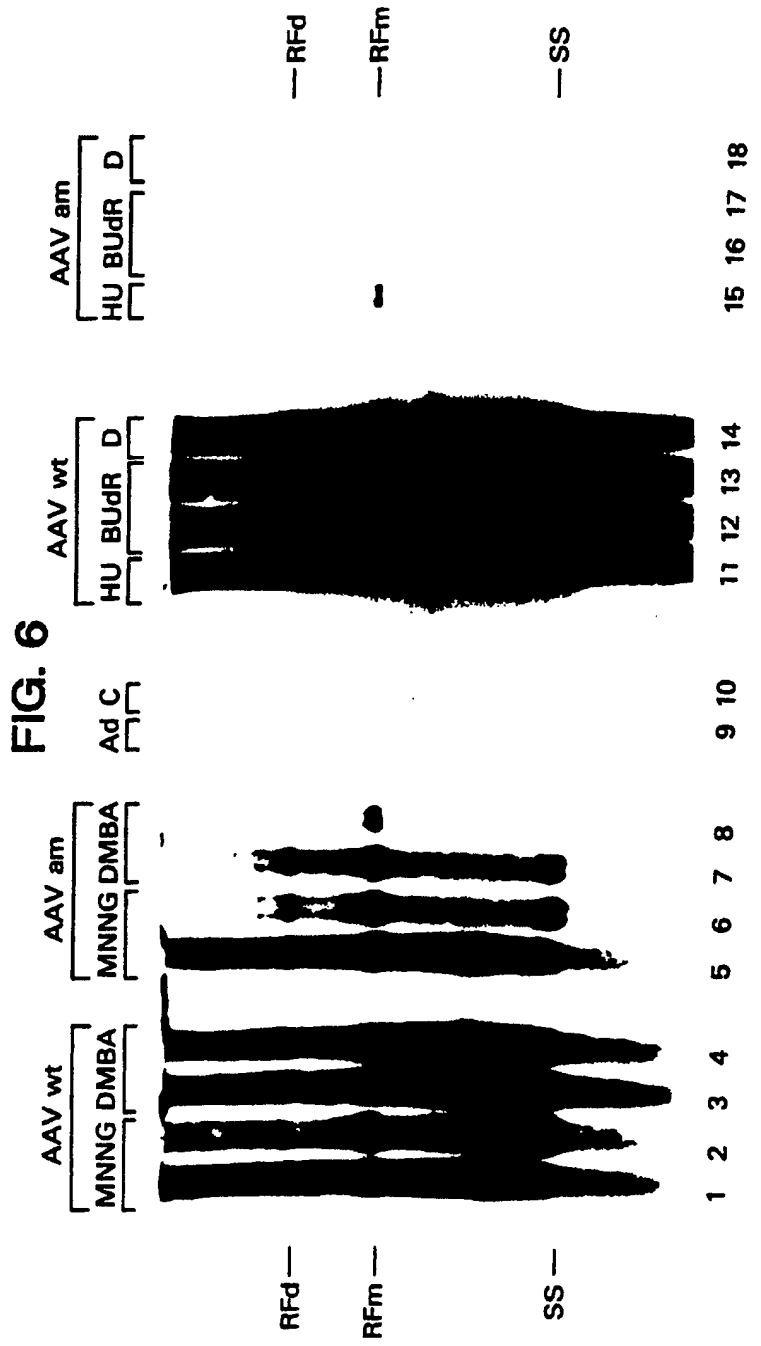

RFd
RFm
SS
AAV am
HU BUdR D
15 16 17 18
AAV wt
HU BUdR D
11 12 13 14
Ad C
9 10
AAV am
MNNG DMBA
5 6 7 8
AAV wt
MNNG DMBA
1 2 3 4
RFd
RFm
SS

FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04765

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/70

U.S. Cl: 435/5

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5; 435/235

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Computerized database search APS and Dialog

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Proceedings of the National Academy of Sciences, U.S.A., Vol. 81, issued October 1984, Paul L. Hermonat et al., "Use of adeno-associated virus as a mammalian DNA cloning vector: Transduction of neomycin resistance into mammalian tissue culture cells", pages 6466-6470.	1-5

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 August 1991	24 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Bradley L. Sisson

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Proceedings of the National Academy of Sciences, U.S.A., vol. 79. issued March 1982, R.J. Samulski et al. "Cloning of the adeno-associated virus into pBR322: Rescue of intact from the recombinant plasmid in human cells", pages 2077-2081	1-5
A	Gene, volume 23, issued 1983, Catherine A. Laughlin et al, et al., "cloning of infectious adeno-associated virus genomes in bacterial plasmids," pages 65-73.	1-5

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . . , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . . . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	Cell, volume 50, issued 31 July 1987. John M. Sedivy, et al., "An Inducible Mammalian Amber Suppressor: Propagation of a Poliovirus Mutant", pages 379-389.	1-5